We now demonstrate, to the best of our knowledge for the first time by direct observation, that anaerobically drawn arterial blood of sickle patients shows birefringence in many red cells and therefore RAPs exist, which we confirm by EM observation of aligned polymers. RAPs exist not only under hypoxemic conditions, when they can be explained by limited solubility due to the presence of deoxyHbS, but also when hypoxemia is absent. RAPs without hypoxemia imply that slow depolymerization kinetics are responsible. One minute of voluntary hyperventilation and (separately) brief nasal oxygen greatly decrease RAPs. RAPs increase during sleep. We attribute these results to accelerated depolymerization at increasing levels of oxygen that cooperatively induce polymer fracture (fracture, using CO, exhibits a 4.7 power dependence on pCO). These results and the interdependent progress of oxygen saturation, partial pressure, fracture rate and remaining polymer that we model bear on pathogenesis and particularly on vaso-occlusive crises, which result from red cell rigidification and from cellular adhesion due to polymer-dependent cellular damage. Under these mechanisms, the lungs may play an important role in initiating pathology; and remediation of dysfunction by breathing assists is potentially prophylactic.

2873-Pos Board B28

Sickle Cell Therapy and the Kinetics of Polymerization in the Presence of Ligands

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Sickle cell disease results from a mutation of normal human hemoglobin that renders it capable of polymerization once oxygen is delivered. The process of deoxygenation involves quaternary as well as tertiary changes in Hb, and both changes appear to be involved in permitting HbS to assemble into the stiff polymers that distort cells and lead to the occlusion of the microvasculature. Kinetics are central to the disease, because if polymerization is slow enough to occur in the veinous return, the polymerization can be reversed upon reoxygention with minimal if any consequences. We have recently completed a detailed study of the polymerization kinetics of HbS in the presence of ligands. We have found that the kinetics are consistent with equilibrium measurements that show singly ligated HbS molecules will polymerize with only about 0.35 the probability of a deoxy HbS molecule. Given knowledge of these highly concentration sensitive rates, and a distribution of intracellular concentrations, we can calculate the likelihood of sickling at various points in the microcirculation. Because of the finite rate of oxygen delivery, we find sickling is most likely at the exit of the capillaries, which is where obstruction has been observed with intravital microscopy. When this is combined with the results for mixtures of fetal hemoglobin (HbF) or HbA, it becomes possible to determine therapeutic targets for the reduction of rates of sickling.

2874-Pos Board B29

Biophysical Studies Evaluating the Potential Physiological Relevance of the Hemoglobin Associated Nitrite Anhydrase Reaction as a Pathway to Generate S-Nitrosothiols from Low Levels of NO and Nitrite

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Hemoglobin (Hb) has been implicated in nitrite-mediated mechanisms that generate bioactive forms of nitric oxide by the reaction of deoxy Hb with nitrite to produce NO (Nitrite Reductase) is viewed as part of the mechanism since the generated NO is readily scavenged, raising questions as to how free NO could escape from the red blood cell. A proposed nitrite anhydrase reaction (NA) between met-Hb and both nitrite and NO to yield N_2O_3, a potent S-nitrosating agent capable of generating longer lived S-nitrosothiols, could address this limitation. Concerns regarding the physiological relevance of the NA reaction stem from the low affinity binding of nitrite to met-Hb and the competition of reductive nitrosylation which generates NOHb. We have identified a relatively stable spectroscopically distinct species generated from met-Hb and the combination of NO and nitrite that can S-nitrosate glutathione. We have tentatively assigned this species to the purported NA intermediate in which N_2O_3 is bound to the heme. The intermediate can be efficiently generated under conditions of low NO and low nitrite. We find that when NO binds to met- Hb, the affinity for the subsequent binding/reaction of nitrite dramatically increases. using sol-gel matrices to trap R and T forms of Hb, we find that for the T state the reductive nitrosylation pathway is favored, whereas for the R state the NA pathway is favored implying a control mechanism for the production of S-nitrosothiols via the NA pathway. Similar studies using HbE, a mutant Hb having an enhanced redox potential, support a mechanism whereby the R/T dependent redox potential is the primary factor that controls the partitioning between the RN and NA reactions of Hb.

2875-Pos Board B30

Modulation of Nitric Oxide Reactivity by Heme Posttranslational Modification in the Cyanobacterial Hemoglobin, GlbN

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Synechococcus sp. PCC 7002 is a model cyanobacterium capable of thriving under conditions that promote the buildup of reactive oxygen and nitrogen species (ROS/RNS). This organism harbors a hemoglobin (GlbN) that is thought to aid in the detoxification of RNS including NO.

GlbN achieves hexacoordinate heme (FeIII/FeII) binding using His70 (proximal) and His46 (distal). In vitro, this coordination scheme protects against H2O2-induced damage, facilitates electron transfer (ET), and lowers redox potential. Under reducing conditions, His117 attacks the 2-vinyl group to form a covalent crosslink. The irreversible posttranslational modification (PTM) of GlbN yields GlbN-A. Ligands such as CO, O2, and NO inhibit the facile PTM. This and other observations suggest that both GlbN and GlbN-A are active in the cell. How does the His-heme PTM influence GlbN reactivity towards NO?

NMR and optical spectroscopies were used to study the differences in NO binding, NO oxidation, ET, and NO reduction. We observed that GlbN and GlbN-A can form unusually stable diamagnetic FeIII-NO complexes. Both FeII-O2 proteins appear capable of NO dioxygenation, where ET is typically rate-determining. Each GlbN exhibits facile ET, with measured self-exchange rates slightly slower than cytochrome b5. A difference in NO reactivity is observed under strongly reducing conditions: surprisingly, unmodified GlbN is capable of reducing NO to nitrosyl hydride (HNO). Additionally, FeII-NO binding in GlbN results in immediate heme loss.

The results provide some insight into the ability of GlbN to protect the cyanobacterium from RNS/ROS. The data suggest that GlbNs can serve as NO dioxygenases, but may not require a dedicated reductase because of their propensity for facile ET. Additionally, an unusual NO reductase-like activity may also exist for GlbN, and the His-heme PTM appears to eliminate this chemistry.

Supported by NSF MCB 0843439

2876-Pos Board B31

Redox-Controlled Proton Gating in Bovine Cytochrome *C* Oxidase

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Cytochrome *c* oxidase is the terminal enzyme in the electron transfer chain of essentially all organisms that utilize oxygen to generate energy. It reduces oxygen to water and harnesses the energy to pump protons across the mitochondrial membrane in eukaryotes and the plasma membrane in prokaryotes. The mechanism by which the oxygen reduction reaction is coupled to proton pumping remains unresolved, owing to the difficulty of visualizing proton movement within the massive membrane-associated protein matrix. Here, with a novel hydrogen/deuterium exchange resonance Raman spectroscopy method (1), we have identified two critical elements of the proton pump: a proton loading site near the propionate A group of heme *a*, which is capable of transiently storing protons uploaded from the negative-side of the membrane prior to their release into the positive-side of the membrane and a conformational gate that controls proton translocation in response to the change in the redox state of heme *a*. These findings form the basis for a new molecular model describing the mechanism, by which unidirectional proton translocation is coupled to electron transfer from heme *a* to heme a_3 associated with oxygen chemistry occurring in the heme a_3 site during enzymatic turnover.

2877-Pos Board B32

Reduction of Iron Center Enhances the Mechanical Stability of Cytochrome B562

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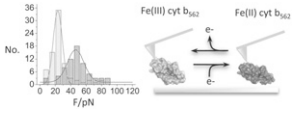
Because redox energy can be converted into conformational energy, heme redox proteins offer a unique opportunity to examine the coupling between redox reactions and protein mechanics. Here, we use Atomic Force Microscopy-based single-molecule force spectroscopy (SMFS) to directly examine the effect of heme and its oxidation state on the mechanical properties of cytochrome b562 (cyt b562). We found that cyt b562 is mechanically stronger in its reduced state as compared to its oxidized state. In addition, we discovered the shortening of



the folded length of cytochrome b562 in its reduced state. This novel observation could be the basis of heme protein-based ''piezoelectric-like'' nanomaterials and actuators.

2878-Pos Board B33

Identification of Electron Transfer Complexes between Shewanella Oneidensis Cytochrome C Peroxidase and ScyA through Computational and Biochemical Analysis

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Bacterial diheme cytochrome c peroxidases form part of the detoxification pathways in the periplasmic space that convert hydrogen peroxide to water. The turnover of peroxide requires a two-electron reduction, and these reducing equivalents must be delivered to the peroxidase by soluble electron transfer proteins. This study focuses on the electron transfer complexes formed between the diheme cytochrome c peroxidase from Shewanella oneidensis (So CcP) and its native electron donor ScyA. Due to the transient nature of this interaction and its presumed dependence on redox state of the two binding partners, traditional biophysical analysis of this system has proved challenging. Here, we report the use of RosettaDock to predict the protein-protein interaction surfaces on So CcP when in the inactive, oxidized state and the active, semi-reduced state. We validate these models by generating site-directed mutants designed to interfere with specific hydrophobic interfaces suggested in the docking analysis and measuring their kinetic competence for both single-electron reductive activation and two-electron peroxide turnover. These results suggest how redox-driven changes in So CcP conformation regulate protein-protein interactions.

2879-Pos Board B34

Probing Substrate Access Pathways of the Active Site of Cytochrome P450 3A4 in its Membrane-Bound Form

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Cytochrome P450 proteins (CYPs) constitute a large group of enzymes involved in the metabolism of a large number of endogenous compounds and xenobiotics. In humans, CYP3A4 is the most abundant isoform, which is responsible for the metabolism of more than 50% of clinically used drugs. It has been suggested that the interaction of CYPs with the membrane is crucial for the binding of liposoluble substrates to the active site of the enzyme.

Molecular dynamics simulations using a HMMM membrane model have captured spontaneous insertion of the globular domain of CYP3A4 into the membrane and revealed a significant reconfiguration of the access tunnels leading to the active site upon membrane binding. We have identified that the binding to the membrane favors the opening of several access tunnels to the active site not observed in the crystallographic structures. Rearrangement of a Phe-cluster appears to be the main membrane-induced event resulting in the opening of the access tunnels. Given the location of these tunnels, they might be involved in leading compounds in or out of the active site. In order to characterize the role of these access tunnels in access/egress of compounds, we have also performed steered molecular dynamics simulations to pull progesterone from the active site of the membrane-bound CYP3A4. Parameters for progesterone were optimized employing the Force Field Toolkit recently developed by our group. We tested 5 different tunnels, previously identified to be open when CYP3A4 is bound to the membrane. To complement the SMD simulations, the partitioning of progesterone into the membrane was also studied. The results of these simulations allow us to elucidate the role of the membrane in the access/egress of compounds to the active site of CYP3A4.

2880-Pos Board B35

Comparison of the Crystal and Solution Structures of a Cyanobacterial Hemoglobin

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GlbN is a monomeric 2/2 hemoglobin found in several cyanobacteria. Physiological data suggest that, in *Synechococcus* sp. PCC 7002, GlbN protects the cell from damage associated with nitrate metabolism [1]. Under reducing conditions, GlbN undergoes a facile posttranslational cross-linking between His117 and the heme 2-vinyl. In a study of the enzymatic and chemical properties of GlbN, the solution structure of the protein in the ferric, bis-histidine state with heme posttranslational modification was determined by NMR methods. As this protein is paramagnetic (S = 1/2) and can be prepared in the reduced state (S = 0), heme methyl hyperfine chemical shifts were used to determine the orientation of the ligating histidines, and proton pseudocontact shifts were applied in the refinement. The NMR model contains regions that appear to sample multiple conformations, specifically the A helix and EF loop. Here we present the X-ray structure of GlbN. Crystals were obtained by the vapor diffusion hanging drop method, and data were collected under cryogenic conditions (National Synchrotron Light Source, BNL). The structure of the related *Synechocystis* sp. PCC 6803 GlbN (1RTX) was used as the search model for molecular replacement. Iterative model building yielded a final structure at 1.65 Å resolution. The X-ray data revealed a ruffled heme, provided a reliable representation of its environment in solution, and allowed for an assessment of the paramagnetic NMR approach. The X-ray data also gave an indication of the effect of crystal packing on the flexible regions of GlbN. Comparison with the *Synechocystis* GlbN structure highlighted the resilience of the 2/2 globin fold as well as subtle differences in the solvent accessibility of the heme active site.

[1] Scott et al. (2010) Biochemistry 49:7000

Supported by NSF MCB 0843439

2881-Pos Board B36

Hemoproteins Designed from Scratch for O2 Transport, Co, No Signaling, Oxidoreductase and Cytochrome P450 Oxygenase Catalysis

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We have designed and engineered a non-natural heme binding protein maquette with diverse heme ligations (bis-his, his-cys, single cys) and dramatically different spectroscopic and redox properties. Hemoproteins in nature perform diversified functions such as oxygen storage, oxygen transport, CO, NO signaling, gene regulation, drug metabolism. Hemoproteins achieve these diversified functions by modifying the redox properties of heme prosthetic group and by altered ligating amino acid to the central Fe metal or by use of different tetrapyrroles or by the nature of amino acids in the vicinity of heme. We have successfully designed a single chain tetra-helix bundle hemoproteins with bis-his, his-cys and single cys ligations, all display native absorption spectra of neuroglobin, CooA (transcription activator), other CO sensing enzymes and cytochrome P450 respectively. All these designed proteins form stable oxyferrous state with natural and synthetic iron porphyrins. These protein maquettes are readily expressable in E.coli. and holds great promise for doing P450 kind of reactions.

2882-Pos Board B37

Engineering a Heme-Protein Crosslink in 2/2 Hemoglobins

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Essentially all hemoglobins (Hbs) bind *b* heme reversibly, but notable exceptions are found in cyanobacterial 2/2 (''truncated'') Hbs. In the Fe^{II} state, the Hb from *Synechocystis* sp. PCC 6803 (GlbN) undergoes a posttranslational modification (PTM) in which a non-iron-coordinating histidine (H117) adds across the heme 2-vinyl. Heme PTMs alter the chemical properties of the prosthetic group and, as such, warrant investigation. The rarity of the histidine-heme crosslink in heme proteins adds interest to the PTM determinants. Here, we examined the robustness of the nucleophilic addition with amino acid replacements. We applied absorbance and NMR spectroscopies to characterize the products.

We generated two GlbN variants. The first, H117A/L79H GlbN, was designed to eliminate PTM at the 2-vinyl and explore reactivity on the 4-vinyl side of the heme. Insertion of a histidine at position 79 caused a severe decrease in ferric heme affinity. Despite this, reduction to the Fe^{II} state resulted in a PTM identical to that observed with H117 in the wild-type protein. Remarkably, the second variant, L79H GlbN, exhibited reactivity at both the 2- and 4-heme vinyls, demonstrating a lack of stringent electronic requirements for the reaction.

To test crosslink formation further, we used the 2/2 Hb from *Chlamydomonas eugametos* (Ce-Hb), which compared to GlbN has a similar fold, only 45% sequence identity, and distinct heme environment in the ligand-free state. We engineered histidine residues at the topological equivalents of H117 and L79 in GlbN (T111 and L75). With changes to the GlbN protocol involving the distal ligand, evidence for reductive heme modification was detected in both Ce Hb variants. The data suggested strongly that properly positioned histidines can cause heme PTM in hemoglobins.

Supported by NSF MCB 0843439